Acetylcholine and calcium signalling regulates muscle fibre formation in the zebrafish embryo

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Summary

Nerve activity is known to be an important regulator of muscle phenotype in the adult, but its contribution to muscle development during embryogenesis remains unresolved. We used the zebrafish embryo and in vivo imaging approaches to address the role of activity-generated signals, acetylcholine and intracellular calcium, in vertebrate slow muscle development. We show that acetylcholine drives initial muscle contraction and embryonic movement via release of intracellular calcium from ryanodine receptors. Inhibition of this activity-dependent pathway at the level of the acetylcholine receptor or ryanodine receptor did not disrupt slow fibre number, elongation or migration but affected myofibril organisation. In mutants lacking functional acetylcholine receptors myofibre length increased and sarcomere length decreased significantly. We propose that calcium is acting via the cytoskeleton to regulate myofibril organisation. Within a myofibre, sarcomere length and number are the key parameters regulating force generation; hence our findings imply a critical role for nerve-mediated calcium signals in the formation of physiologically functional muscle units during development.

Supplementary material available online at
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Key words: Activity dependent, Muscle development, Acetylcholine, Calcium, Sarcomere, Zebrafish

Introduction

Proper skeletal muscle function is critical for coordinated movement. The foundations of the skeletal muscle system are established in the embryo where myogenesis and the specification of muscle fibre types are known to be regulated via a combination of endogenous and exogenous cues. Different muscles have different properties (e.g. size and the assembly of contractile units) which determine their function, but very little is known about developmental factors that regulate the later stages of muscle formation. Nerve input is a central regulator of fibre phenotype in mature tissues (Gunderson, 1998). In the embryo, once functional neuromuscular contacts form, motor neurons have the potential to regulate muscle differentiation. Indeed innervation is capable of regulating the expression of muscle-specific proteins during development (DiMario and Stockdale, 1997; Leberer et al., 1986; Washabaugh et al., 1998) and can influence the size of the muscle (Misgeld et al., 2002). This raises the possibility that nerve activity also regulates the formation and/or assembly of functional contractile units at later stages of myogenesis.

In mature muscle, calcium acts as a messenger linking excitation events at the membrane with downstream effects, such as contraction, ATP production and transcription (Berchtold et al., 2000). Muscle cells are capable of generating calcium signals early in their development, even in the absence of innervation (Bakker et al., 1996; Ferrari and Spitzer, 1999; Flucher and Andrews, 1993; Lorenzon, et al., 1997). Indeed nerve-independent calcium signals have a role in somite maturation and myofibrillogenesis (De Deyne, 2000; Li et al., 2004). While myocytes and nerve-muscle cocultures remain an excellent model for examining signalling at a cellular level, only analysis in vivo permits the examination of nerve generated calcium signals in muscle cells subjected to the full range of developmental cues. The contribution of nerve activated calcium signals to muscle fibre development in intact embryos remains undefined. Here we use the zebrafish embryo as an in vivo model to address the role of activity dependent signals, acetylcholine and calcium, in later stages of muscle development.

The zebrafish embryo is an important organism for the study of vertebrate muscle development (Brennan et al., 2002; Stickney et al., 2000). The anatomical development of the early neuromuscular system in the zebrafish embryo has been well described (Fig. 1). The earliest movements of the zebrafish embryo (side to side coiling of the tail) at 17 hours post-fertilisation (hpf) are an external indication that nerve-muscle contact has occurred (Kimmel et al., 1995; Saint-Amant and Drapeau 1998). Activity-dependent muscle contractions are generated from the outset via cholinergic activation (Melancon et al., 1997). Analysis of zebrafish mutants (*ache* and *twister*) has also implicated cholinergic neurotransmission in slow muscle fibre formation and degeneration (Behra et al., 2002; Lefebvre et al., 2004). The intracellular signalling mechanisms that are activated in neurotransmitter-regulated muscle development are unknown. We set out to address the activation

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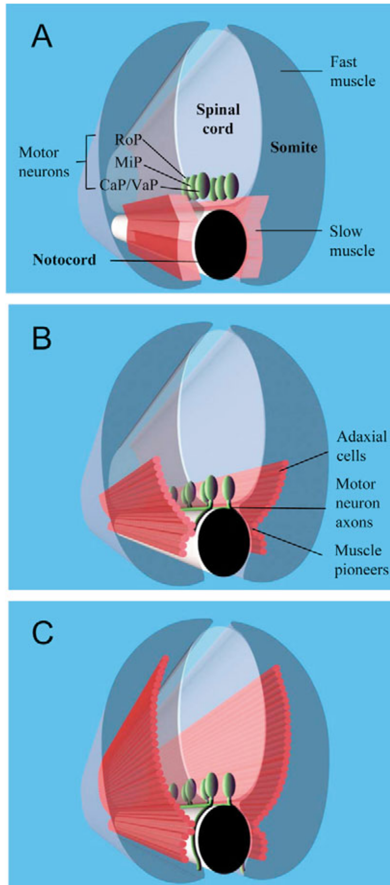

Fig. 1. Early development of the neuromuscular system in the trunk of the embryo. (A) At 16 hpf. In each spinal cord segment three or sometimes four motor neurons (RoP, MiP, CaP/VaP, as shown in green) are positioned ventrally either side of the midline (Eisen, et al., 1986). In the somite there are two populations of muscle cells, adaxial cells (shown in red) and lateral presomitic cells that differentiate into slow fibres and fast fibres respectively (Devoto et al., 1996). At around 18 hpf the motor neurons begin axon extension. Axons leave the spinal cord at a common point and grow along the medial surface of the somite (Eisen et al., 1986). Cuboidal adaxial cells, arranged in a 4×5 array adjacent to the notochord, begin to elongate and migrate within the somite (Devoto et al., 1996). (B) At 19 hpf. The leading motorneuron axon makes contact and pauses at a specialized set of muscle fibres, termed muscle pioneers. The muscle pioneers, a subset of adaxial cells that remain adjacent to the notocord, become flattened cells separating the dorsal and ventral somite. Adaxial/slow muscle fibres have formed a 1×20 array of elongated muscle fibres. (C) At 22 hpf, motor neuron axon pathways now diverge to innervate specific areas of the somite. Adaxial cells migrate radially towards the lateral surface of the somite (Devoto et al., 1996).

of intracellular messengers downstream of acetylcholine and their potential role in regulating muscle development. In mature muscle, acetylcholine mediates its effects via acetylcholine receptor (AChR) induced increase in intracellular calcium. The zebrafish embryo has proved extremely useful for imaging intracellular calcium signals in situ (Ashworth, 2004). In the present study we characterize the generation of the earliest endogenous activity-dependent calcium signals in the muscle fibres of intact zebrafish embryos and investigate their role in muscle development. Inhibition of acetylcholine and ryanodine receptors revealed a role for activity-dependent signalling pathways in the regulation of myofibril bundling, sarcomere length and fibre length in slow muscle fibres. Taken together these results reveal that nerve activity plays a vital role in muscle fibre formation and function within the embryo.

Materials and Methods

Fish maintenance

Wild-type zebrafish (*Danio rerio*) were maintained as described previously (Ashworth et al., 2001). Mutant lines *nic1^{b107}* (*nic1*) were obtained from the Zebrafish International Resource Center at the University of Oregon. Heterozygote adults were crossed to obtain homozygous embryos, identified by lack of movement after 17 hpf. Staging of the embryos was performed as described by Kimmel and colleagues, and is given in the text as standard developmental time (hpf) (Kimmel et al., 1995).

Intracellular calcium measurements during muscle fibre contraction

Eggs were injected as described previously (Ashworth, 2004). The high affinity calcium indicator Oregon Green 488 BAPTA-1 dextran (10 kDa), with a K_d=265 nM and the low affinity calcium indicator Fluo-4 dextran (10 kDa), with a K_d of 3 μM were used to detect changes in cytosolic calcium in the nM and μM range, respectively (Molecular Probes) (Haugland, 2002). The use of single wavelength calcium indicators to measure calcium fluxes presents the possibility of artefacts in fluorescence intensity changes, the most obvious in the present study being significant cell movement during muscle contraction (Fetcho et al., 1998; Lipp and Niggli, 1993). In the present study we have loaded cells with the calcium indicator (either Oregon Green BAPTA dextran or Fluo-4 dextran) in combination with the calcium insensitive fluorescent dye tetramethylrhodamine dextran (10 kDa; Molecular Probes). The assumptions are that: (1) the dyes have a good spectral separation (note that sequential imaging was performed to reduce signal overlap); (2) no significant bleaching was occurring; (3) that the dyes display a homogeneous distribution; and (4) that there is no compartmentalization (the use of dextran conjugate ensures the indicator is retained within the cytosol thus avoiding the problems of compartmentalisation associated with calcium indicators). During the recording the cells of interest were focused in the brightest plane, resulting in a decrease in fluorescence during movement but an increase in intensity due to changes in the calcium signal (Ashworth et al., 2001; Fetcho et al., 1998). Thus signal from the rhodamine will reflect changes in fluorescence due to movement and not changes in calcium, and provides a method of obtaining ratiometric measurements (supplementary material Fig. 1B,C). The K_d of Fluo-4 dextran means that the indicator does not emit a fluorescent signal in resting cells where the cytosolic calcium ion concentration will be in the nM range. Therefore, in the experiments using Fluo-4 dextran, fibres were identified from the signal emitted by excitation of rhodamine dextran. The dyes were loaded into cells to give an estimated intracellular concentration of 40-80 μM previously shown not to disrupt cellular development in neurons (Ashworth et al., 2001). The use of the higher affinity indicator Fluo-4 at the same concentration is unlikely to buffer calcium signals.

Fluorescence imaging was performed on a confocal laser-scanning microscope (Zeiss LSM 510). All recordings were performed at room temperature (22°C). Embryos were mounted in 1% agar solution and orientated horizontally to obtain a side view. Specimens were viewed through a Zeiss Fluar ×20 objective (N.A. 0.75). To capture the cytosolic calcium signals as the first neuromuscular contacts form, dye-labelled muscle fibres located at the nascent horizontal myoseptum (in a position consistent with their identity as a subset of

slow muscle fibres, muscle pioneers) were selected (supplementary material Fig. 1A). Fibres were chosen on the basis that they could be identified as a single fibre that appeared to span the entire somite, and the four most caudal somites were never imaged. Oregon Green 488 BAPTA-1 dextran and Fluo-4 dextran were excited using a 488 nm laser line and collected through a 530-550 nm band pass filter. The rhodamine was excited using a 543 nm laser line and emission collected through a 570 nm long pass filter. To minimize cross talk between the two dyes the signals were collected sequentially. All images were processed and analyzed using Zeiss software. Changes in fluorescence were calculated as the average pixel intensity within user-defined regions drawn around the muscle fibres. Further analysis was performed using Microsoft Excel spreadsheets, and statistical analysis was performed using Graphpad InStat v3.05.

Administration of drugs to embryos

To gain access to internal tissues it was necessary to perform tail cuts on embryos, a procedure that has been described previously (Liu and Westerfield, 1990). For the experiments described in this study a cut was made at the caudal end using a sharp needle, and then embryos transferred to experimental solutions. The drugs used were Rhodamine α-bungarotoxin (Molecular Probes), Dantrolene (Calbiochem), nifedipine and ryanodine (Calbiochem). To determine whether drugs inhibit spontaneous contractions, tail cut embryos (20-20.5 hpf) was incubated in experimental treatments for 30 minutes at room temperature. Each embryo was then assessed over a 5 minute observational period and blockade of contractions was determined by lack of spontaneous movement and failure to respond to touch.

Immunocytochemistry

For immunocytochemistry embryos were fixed and stained as described previously (Ashworth et al., 2001). Primary antibodies were mouse anti-myosin (F59, originally kind gift from F. Stockdale) used at a 1:10 dilution, mouse anti-ryanodine receptors (34C, from DSHB) used at a 1:250, rabbit anti-human prox-1 (from RELIATech GnbH, Germany) used at a 1:500 dilution. The secondary antibodies were Goat anti mouse IgG, Cy-5 linked (Amersham) used at a dilution of 1:1000 and Goat anti rabbit IgG Cy-3 linked (Jackson ImmunoResearch) used at a dilution of 1:500. After incubation with secondary antibodies actin was stained in wholemount embryos using Alexa Fluor 488 phalloidin (Molecular Probes) diluted 1:20 in phosphate buffer for 2.5 hours. Images from whole mount stained embryos were collected using laser scanning confocal microscope (Zeiss LSM 510), using Fluar 20×/0.7 UV and C-Apochromat 63×/1.2 W. Stacks of images were reconstructed in three dimensions to generate lateral and transverse projections.

In situ hybridization and immunohistochemistry

Zebrafish ryanodine receptor fragment clones from the EST database were used to synthesise digoxigenin (DIG)-labelled antisense riboprobeS for in-situ hybridization as described (Thisse et al., 1993). A 1:250-400 dilution of the riboprobe was used to establish embryonic expression. Primary antibody was used at a 1:10 dilution (pan muscle myosin A41025; gift from Simon Hughes) and secondary antibodies at a 1:600 dilution (monoclonal anti mouse IgG; Calbiochem). For cryostat sectioning wholemount stained embyos were embedded in low-melting-point agarose, equilibrated in 30% sucrose/PBS solution and sectioned at 15 microns (Leica cryostat).

Sequence alignment

Plasmid clone (019-D04-2, Acc No AL916615) obtained from Peng Jinrong, Functional Genomics Laboratory, Institute of Molecular and Cell Biology, National University of Singapore, 30 Medical Drive, Singapore 117609. The nucleotide sequence for clone 019-D04-2 was used to search the zebrafish genomic database and produced a 100% match with the coding sequence from genomic clone BX682544.5 from base 14023 to base 14595. Translation of the full-length coding sequence from BX682544.5 produced a protein sequence that was used to generate a multiple alignment using ClustalW (http://www2.ebi.ac.uk/clustalw/). Multiple alignment protein parameters used default parameter settings: gap opening 75.00, extension 0.30. A phylogenetic tree (dendogram) was created based on this ClustalW generated alignment (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

TEM

Embryos for TEM were dechorionated, wild type anaesthetized in Tricaine (MS-222) and fixed in 0.1 M sodium cacodylate, 0.5% calcium chloride, 2% paraformaldehyde and 2.5% glutaraldehyde. After fixation, embryos were rinsed in 0.1 M sodium cacodylate, post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate, rinsed in H_2O and then incubated in 2% uranyl acetate in H_2O for 15 minutes. Specimens were further washed in H_2O before being dehydrated through a graded ethanol series. Embryos were embedded in Agar Resin mix and ultrathin sections were cut. Sections were examined using a Jeol 1010 transmission electron microscope.

Results

Activity-generated cytosolic calcium transients in slow muscle fibres display a characteristic developmental pattern

Variation in the dynamics of intracellular calcium signals (such as amplitude and duration) is a key mechanism by which downstream responses to calcium signalling are regulated (Berridge et al., 2000; Dolmetsch et al., 1997). In muscle, the pattern of nerve-mediated depolarisation influences expression of contractile proteins (DiMario and Stockdale, 1997; Washabaugh et al., 1998). This raises the possibility that variation in nerve-mediated calcium signals represents a mechanism by which nervous activity may regulate muscle development. To begin to address whether nerve activity may regulate embryonic muscle development we characterised the dynamics of nerve generated calcium signals in muscle cells during initial stages of slow muscle development following initiation of motor neuron contact. We recorded changes in intracellular calcium from individual fibres in intact zebrafish embryos, as described previously (Ashworth, 2004) (see also supplementary material Fig. S1). Recordings were made between 16 and 22 hpf, at a stage in which functional neuromuscular contacts in zebrafish embryos are first established. The calcium changes are proposed to represent signals from a single population, most likely a subpopulation of slow muscle fibres (see supplementary material Fig. S1). Two indicators with different affinities for calcium ions were used; Oregon Green BAPTA dextran (K_d=265 nM) and Fluo-4 dextran (K_d=3 μM) to detect changes in concentrations in the nM and μM concentration range respectively (see supplementary material Fig. S1). In embryos loaded with Oregon Green BAPTA dextran no changes in cytosolic calcium were recorded in muscle fibres between 16.5 and 17.25 hpf (*n*=4 embryos, Fig. 2A). The first calcium transients were detected at 17.25 hpf and corresponded to the appearance of the first visible fibre contractions in the embryo. The earliest calcium transients exhibited a mean frequency of 0.005 Hz, reaching a maximum of 0.11 Hz at 19 hpf before decreasing

to <0.036 Hz by 22 hpf (Fig. 2A). No changes in the concentration of cytosolic calcium ions were detected in Fluo-4 dextran loaded muscle fibres from embryos aged between 17.25 and 18 hpf (*n*=3 recordings, Fig. 2B). Calcium transients were recorded from Fluo-4 dextran loaded muscle fibres in embryos aged between 18-19 hpf (*n*=6-8 recordings), and 21.5h (*n*=2 recordings). The frequency of the calcium transients recorded using Fluo-4 dextran were not significantly different to those measured with Oregon Green 488 BAPTA dextran at all developmental times measured after 18 hpf (Fig. 2B). The functional maturation of the neurotransmitter evoked calcium signals were investigated by examining the onset and decay of individual transients. The mean average time for the signal to rise from the baseline to the peak, 0.645±0.05 seconds (*n*=21), did not change significantly with developmental age between 17 and 21 hpf (Spearman Rank Correlation, *r*=–0.46, data not shown). The rise in the cytosolic calcium ion concentration in the muscle fibre appeared maximal from the start, at least at the temporal resolution of our measurements (approximately 380 milliseconds). A significant (6-fold) decrease in the decay time of the calcium signal from 3.0 to 0.5 seconds was observed in the 3 hour developmental period between 18 and 21 hpf (Fig. 2C) (Spearman Rank Correlation, *r*=–0.825, *P*<0.0001). Our results show that the acetylcholine generated calcium signals in slow muscle fibres appear in three distinct phases (summarised in Fig. 2D) during the initiation of neuromuscular contact. The spatial and temporal dynamics of intracellular calcium signals are critical for the selective activation of downstream effectors (Berridge et al., 2000). The developmentally regulated variation in calcium dynamics is consistent with nerve-mediated calcium signals directing specific components of slow muscle development.

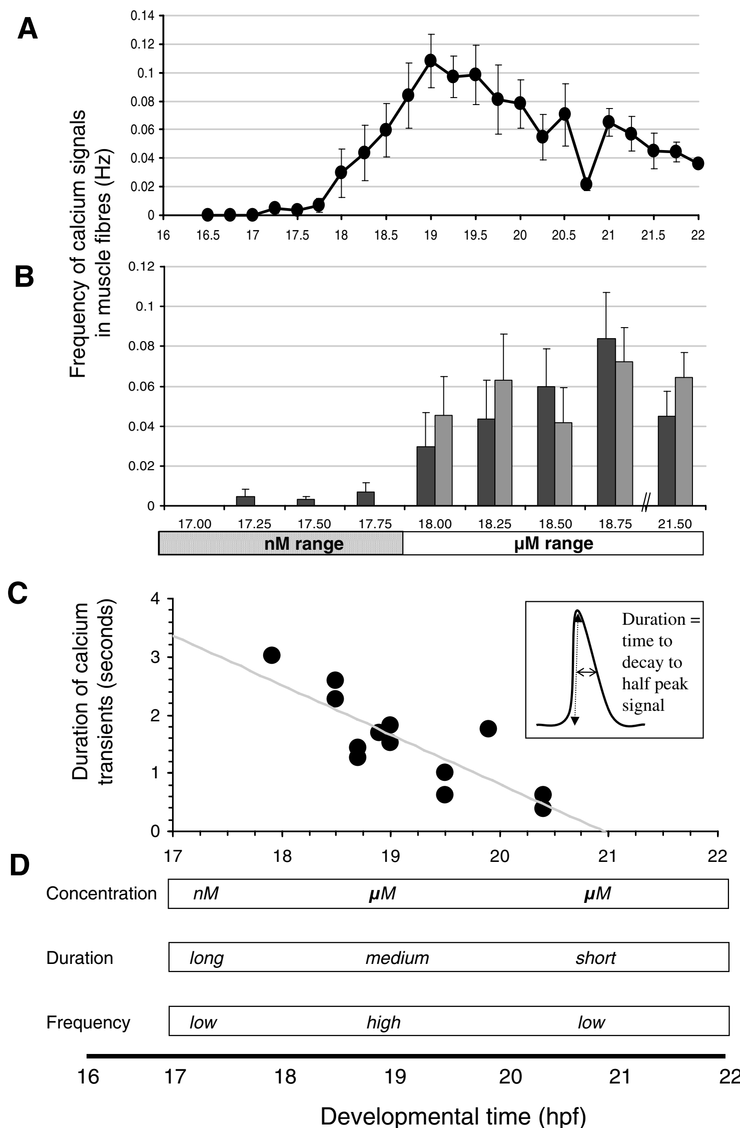

Fig. 2. Acetylcholine-generated calcium transients in muscle fibres within the embryo. (A) The frequency of the calcium transients (mean ±s.e.m.), recorded from whole muscle fibres using Oregon Green 488 BAPTA dextran, was plotted against standard developmental time (hpf). (B) The frequency of the calcium transients (mean ±s.e.m.) measured using the high affinity Oregon Green 488 BAPTA dextran (black bars, nM range) compared with the lower affinity Fluo-4 dextran (grey bars, µM range) were plotted against standard developmental time (hpf). There was no significant difference between the frequency of the calcium signals measured with two indicators between 18 and 19 hpf, or at 21.5hpf (unpaired *t*-test). (C) Traces (*n*=13) were selected in which fluorescence changes, reported using Oregon Green BAPTA dextran, correspond to changes in cytosolic calcium ion and not to cell movements (see supplementary material Fig. S1). The duration of the signals, defined as the time taken to decay from maximum amplitude to half that value (see inset) decreased significantly between a developmental period of 18-21 hpf (Spearman Rank Correlation, *r*=–0.825, *P*<0.0001). (D) Calcium signals in the anterior trunk axial muscle display a characteristic developmental pattern between 16 and 22 hpf.

Activity-generated calcium signals in embryonic muscle are generated via L-type calcium channels and ryanodine receptors

Postsynaptic calcium signals in vertebrate skeletal muscles are generated via mechanical coupling of specialized calcium channels, namely dihydropyridine (DHPR) in the sarcolemma and ryanodine receptors (RyR) in the sarcoplasmic reticulum. To establish the involvement of these proteins in early nerve-mediated calcium signals in zebrafish slow muscle, initially we characterised the developmental expression of zebrafish RyR using in situ hybridisation and immunohistochemistry. We used pharmacological blockade with nifedipine and dantrolene to demonstrate a functional role of DHPR and RyR respectively.

The ryanodine receptor in particular has been implicated in myotome development (Ferrari and Spitzer, 1999). We examined the expression patterns of available zebrafish RyR EST clones showing a high homology with other vertebrate RyR by mRNA in situ hybridisation (Fig. 3 and data not shown). The RyR3 clone (019-D04-2) was expressed in the developing somites, specifically in the adaxial slow muscle precursor cells from the four somite stage (data not shown). At the 10 somite stage RyR3 expression was detected in the muscle pioneer and adaxial cells of all

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formed somites and within the lateral somitic mesoderm of the more mature somites (Fig. 3C). At 24 hpf, expression was detected throughout the somitic mesoderm in both fast and slow muscle populations (Fig. 3D,E). Elevated levels of expression in the most dorsal domain of the somite corresponded to the dorsal growth zone of the somite (Barresi et al., 2001). Somite specific expression of RyR3 got stronger as the embryo matured from 16-24 hpf up to 48 hpf. In mammals three isoforms of the ryanodine receptor (1, 2 and 3) have been cloned and sequenced. RyR3 is expressed in mouse foetal and neonatal tissues and has a role in amplification of the calcium signal during the perinatal period (Bertocchini et al., 1997). We show that ryanodine receptor mRNA, analgous to vertebrate RyR3, is expressed exclusively in skeletal muscle of the zebrafish embryo from early somite formation onwards.

The clustering of ryanodine receptor protein is a critical step in the maturation of calcium release units in muscle (Takekura et al., 2001). We examined the developmental distribution of the RyR receptor protein in the somites using whole mount immunocytochemistry. Clusters of ryanodine receptors were observed throughout the trunk somites from 20 hpf onwards (Fig. 3F,G). Neural signals are not required for the developmental induction of ryanodine receptor gene expression (Radzyukevich et al., 2004). To determine whether neural signals are required for clustering of RyR receptors we examined expression in zebrafish mutant *nic1* embryos that lack functional nictonic acetylcholine receptors (AChR) and thus cannot respond to neural signals (Fig. 3H,I) (Sepich et al., 1998; Westerfield et al., 1990). RyR expression was normal in *nic1* mutants indicating that activity is not a requirement for ryanodine receptor maturation and clustering.

Having established that RyR receptors are expressed within the zebrafish slow muscle precursors from the earliest stages of differentiation we adopted a pharmacological approach to demonstrate their functional role. Drugs do not readily diffuse across the skin of later stage embryos but can be introduced via tail cuts (Liu and Westerfield, 1992). The frequency of intracellular calcium signals in muscle fibres of tail cut embryos (0.088±0.004 Hz, *n*=2) was not significantly different from intact embryos (0.082±0.023 Hz, *n*=12, unpaired *t*-test) at 19 hpf. Therefore tail cuts alone do not disrupt the generation of cytosolic calcium transients in the muscle. Tailcut embryos were bathed in relatively high concentrations of drugs to encourage penetration into internal tissues; however the uptake and concentration at the target site could not be measured. Between 20 and 21 hpf the majority (94±3.4%) of embryos display activity dependent spontaneous contractions (*n*=50 embryos). We tested the effect of calcium channel blockers on the generation of activity dependent muscle contraction in embryos. The organic blocker nifedipine targets the dihydropyridine binding site on the α_1 subunit of the skeletal muscle L-type

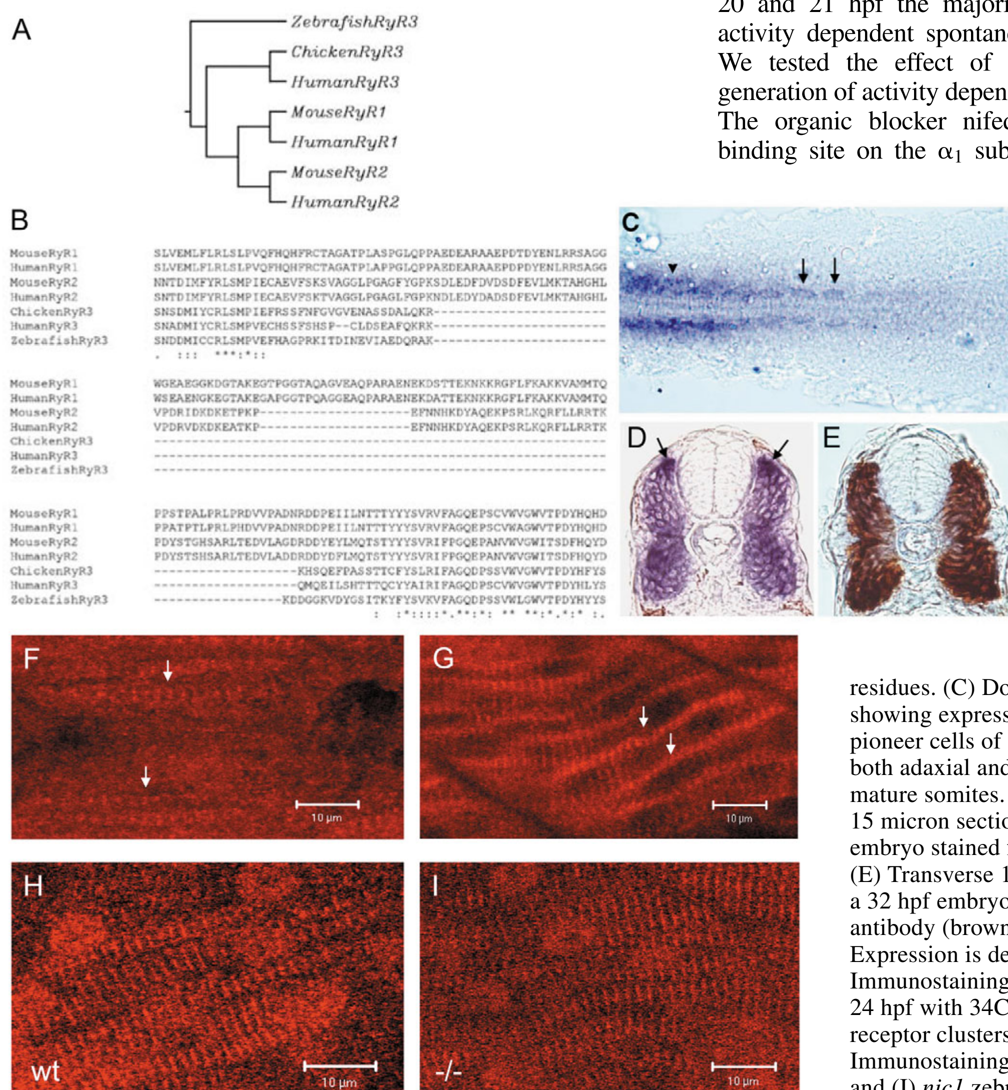

Fig. 3. Expression of ryanodine receptors in embryo. (A) Phylogenetic tree of vertebrate RyR family members based on a ClustalW generated alignment of zebrafish RyR3 (CAI11683), chicken RyR3 (Q90985), human RyR3 (Q15413), mouse RyR1 (Q80X16), human RyR1 (P21817), mouse RyR2 (Q9ERN6) and human RyR2 (Q5VWP1). (B) Sequence alignment of the divergent region showing: *, identical residues; **:**, conservative residues; **.**, semi-conservative residues. (C) Dorsal view of a 10 somite embryo showing expression of *zfRyR3* in adaxial muscle pioneer cells of newly formed somites (arrows) and both adaxial and paraxial (arrowhead) cells of more mature somites. Anterior is to the top. (D) Transverse 15 micron section through the trunk of a 32 hpf embryo stained for *zfRyR3* expression (blue). (E) Transverse 15 micron section through the trunk of a 32 hpf embryo stained with the pan muscle myosin antibody (brown) and for *zfRyR3* expression (blue). Expression is detected throughout the myotome. Immunostaining in embryos aged (F) 20 hpf and (G) 24 hpf with 34C antibody (red) revealed ryanodine receptor clusters from 20 hpf onwards (arrows). Immunostaining using 34C antibody in (H) wild type and (I) *nic1* zebrafish embryos at 48 hpf. Bars 10 µm.

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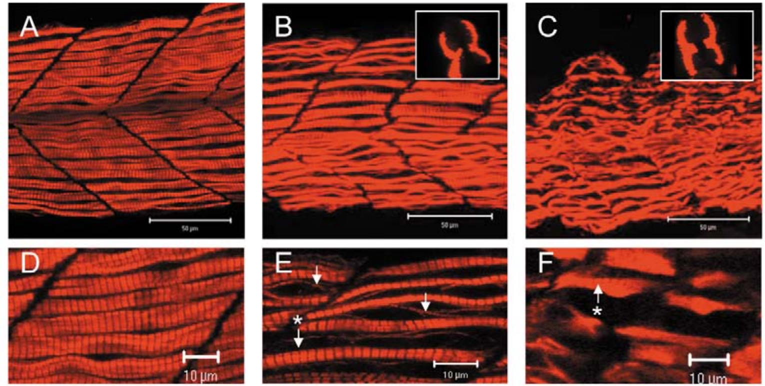

Fig. 4. Slow muscle development is disrupted in ryanodine treated embryos. Embryos were treated with ryanodine just prior to 17 hpf and fixed and stained at 24 hpf. Immunostaining with antibody F59 was performed to reveal slow muscle fibres in (A,D) control (B,E) 10 μM (C,F) 50 μM ryanodine treated embryos. Bars (A-C) 50 μm and (D,E) 10 μm. Insets (B,C) show cross section of slow muscle myosin in anterior trunk of embryos. (E,F) In ryanodine treated embryos striations (arrows with asterisks) were evident and myofibrils (arrows) that had not aligned into bundles were observed.

calcium channel (Zamponi, 1997). Nifedipine treatment, at a concentration of 10 μM and 100 μM, produced a significant dose dependent decrease in the number of contracting embryos (54.54±7.5% n=56 and 58.92±6.57% n=44, respectively, ANOVA P<0.001 compared with controls). Dantrolene and ryanodine can suppress intracellular calcium release from the sarcoplasmic reticulum (SR) by inactivating the ryanodine receptor (Meissner, 1986; Paul-Pletzer et al., 2002). A significant dose dependent decrease in the number of contracting embryos were observed in the presence of 10 μM, 100 μM and 1000 μM Dantrolene (51.85±9.62% n=18, 41.66±10.06% n=24, 38.89±11.49% n=27 respectively, ANOVA, P<0.001 compared with controls). Ryanodine treatment also produced a dose dependent decrease in the number of contracting embryos, at a 10 μM and 50 μM (25±15.3% n=37 and 0% n=25, respectively, ANOVA P<0.001 compared with controls). With the exception of 50 μM ryanodine, muscle contraction was not completely inhibited in treated embryos even at higher drug concentrations. These observations suggest that the calcium signalling pathways that mediate contraction are more complex than originally supposed, consisting of several components perhaps nerve dependent and independent. Our observations in the zebrafish embryo, that application of ryanodine completely abolishes movement, is in contrast to *C. elegans* where intracellular calcium release from ryanodine receptors is not essential for excitation-contraction coupling (Maryon et al., 1996). The pharmacological data we present here suggests that L-type calcium channels and ryanodine receptors contribute to the earliest activity-dependent contractions in zebrafish embryos. Application of drugs to the whole embryo has not allowed us to determine whether the effects are specific to sites at the neuromuscular junction or elsewhere. The expression analysis suggests the involvement of a zebrafish RyR3 homologue in the developing muscle fibres. Immunocytochemistry has revealed that ryanodine receptor protein is expressed and forms clusters in developing muscle fibres. Further identification of the signalling proteins that contribute to the muscle contraction will help in the design of experiments to target the pathway more directly.

Inhibition of ryanodine receptors disrupts the late stages of slow muscle fibre development

Having demonstrated developmentally regulated activity dependent calcium signals, mediated by DHPR and RyR, within the newly formed slow muscle population we investigated the possibility that these calcium signals regulate somite maturation and/or myofibrillogenesis. Calcium signalling in the muscle was blocked by targeting the ryanodine receptor, which plays a major role in the generation of nerve-generated muscle contraction. Embryos were incubated in ryanodine at 16 hpf just prior to the beginning of movements, and fixed for immunostaining of slow muscle fibres at 24 hpf. Lateral migration and elongation are key steps in fish slow muscle development (Stickney et al., 2000). Slow muscle in the anterior somites of zebrafish trunk, assumed to have completed fibre migration by 24 hpf (Stickney et al., 2000), was examined (Fig. 4). Ryanodine treated embryos were found to display a disrupted phenotype by 24 hpf and the trunk of the embryo appeared twisted and shortened (data not shown). In ryanodine treated embryos slow muscle fibres appeared to have elongated in an anterior-posterior direction and migrated to lateral surface; however, the fibre phenotypes appeared progressively disrupted with increasing concentrations of drug (Fig. 4A-C). Somite width in the anterior trunk of embryos treated with 50 μM ryanodine (41.39±2.12 μm, n=20) was very significantly reduced compared with embryos treated with 10 μM ryanodine (56.41±1.56 μm, n=18) and controls (60.66±1.00 μm, n=39, P<0.0001, ANOVA). We suggest that application of high doses of ryanodine may lock the muscle in a contracted state thereby decreasing both somite width and fibre length. Striations, probably corresponding to alignment of sarcomeres, in muscle were evident in both treated and control embryos; however, in ryanodine treated embryos not all the myofibrils had aligned into bundled muscle fibres (Fig. 4D-F).

Nerve activity and downstream signals can influence the proportion of fibre types in mammalian skeletal muscle (Spangenburg and Booth, 2003). We used expression of prox-1 to follow the effect of nerve activity on slow muscle fibre number in embryonic zebrafish. Slow muscle fibres in the zebrafish myotome are mononucleate and express the regulatory protein prox-1 within the nucleus at 24 hpf (Roy et al., 2001). The expression of prox-1 was not disrupted in the presence of ryanodine (data not shown). The number of prox-1 expressing nuclei in dorsal half of the somite was not significantly different in ryanodine treated embryos (50 μM ryanodine, 8±0.2, n=4) compared with controls (9±0.25, n=5). We show that ryanodine receptor activation does not appear to regulate the number of nuclei, gross morphological movements or striation (sarcomere) formation in slow muscle fibres. However, ryanodine receptor activation, and resulting

intracellular calcium release, is required, for myofibril bundling in slow muscle.

Activity-generated signals are required for the organisation of slow muscle myofibrils

Application of ryanodine to the embryo does not allow us to resolve whether our observations on muscle development are a result of direct inhibition within the muscle fibre or secondary inhibition of nervous activity. To determine if activity evoked signals within the muscle fibre regulate development we targeted the acetylcholine receptor. In the zebrafish embryo the first muscle movements occur at 17 hpf and are generated via acetylcholine (ACh) release from motor nerve terminals (Grunwald, et al., 1988; Melancon, et al., 1997). In zebrafish, nicotinic AChR expression and clustering is observed exclusively in somites, beginning around the time of innervation (Liu and Westerfield, 1992). To investigate the role of acetylcholine driven pathways in slow muscle development we have used both pharmacological and genetic approaches. Administration of α-bungarotoxin at the onset of movement (16-17 hpf) inhibited spontaneous contractions in embryos at 24 hpf (*n*=55 embryos). Cytosolic calcium transients in the muscle fibres of embryos aged between 18 hpf and 20 hpf were completely abolished in the presence of 0.5 μM α-bungarotoxin (*n*=18) (Fig. 5A,B). Embryos were fixed at 24 hpf and slow muscle development examined using immunocytochemistry. In α-bungarotoxin treated embryos adaxial cells were observed to have elongated and migrated to form a layer of superficial muscle cells; however, in treated embryos the fibres appear more disorganised (Fig. 5C,D). Thus inhibition of the acetylcholine receptor during neuromuscular development does not affect slow muscle cell migration but disrupts myofibril organisation. To address the role of acetylcholine receptors in more detail we followed up our observations from the pharmacological approach in an established mutant line.

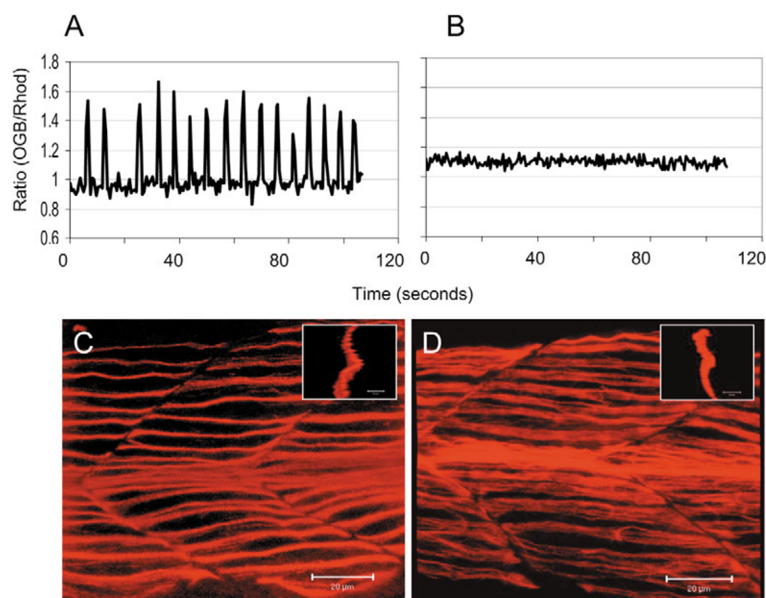

Fig. 5. Acetylcholine generates calcium transients and regulates slow muscle development. Embryos were loaded with Oregon Green BAPTA dextran and incubated in (A) embryo medium or (B) AChR blocker α-bungarotoxin (0.5 μM) for 30 minutes prior to imaging. All calcium signals were inhibited in the presence of the acetylcholine receptor blocker α-bungarotoxin between 18-20 hpf (*n*=18). Tailcut embryos were incubated in (C) embryo medium, or (D) AChR blocker α-bungarotoxin (0.5 μM) from 16 hpf and fixed at 24 hpf. Immunostaining with antibody F59 revealed slow muscle fibres. Inset shows cross section to reveal slow muscle distribution in one half of embryo. Bars 20 μm.

Previously, the role of acetylcholine receptors in development has been studied using the *nic1* zebrafish line that carries a mutation in the α-subunit of the receptor (Sepich et al., 1998; Westerfield et al., 1990). Embryos carrying the *nic1* mutation do not express AChRs and are immotile; however, observations that somite and skeletal muscle fibre formation appears grossly normal suggests that functional acetylcholine receptors are not required for their development. In the present study, we performed a detailed analysis of slow muscle fibre development in *nic1* mutants. By 24 hpf adaxial cells had elongated and migrated to the lateral surface of the somite in both mutant (Fig. 6D-F, *n*=8) and wild-type embryos (Fig, 6A-C, *n*=12); a result indicative that the cholinergic system is not involved in these processes. In the wild-type embryos the myofibrils are packed together into longitudinal bundles to form fibres, while in the mutant embryos myofibrils are not aligned laterally but appear disorganised. Average somite width, taken from measurements in the dorsal half of trunk, was not significantly different in wild type (49.42±1.51, *n*=24) compared with mutants (52.75±1.91, *n*=22, unpaired *t*-test). However, myofibril length relative to somite width was shown to be significantly larger in *nic1* mutant compared with wild-type embryos at 24 and 48 hpf (Fig. 6G). Expression of the homeobox gene *prox-1* required for terminal differentiation of slow muscle fibres was not disrupted in the mutant or in BTX treated embryos (data not shown). There was no significant difference between the number of prox-1 expressing nuclei in the anterior somites of wild type (8±2 nuclei in the dorsal somite, *n*=8 somites from three embryos) compared with homozygote embryos (9±1 nuclei in the dorsal somite, *n*=9 somites from three embryos). Dystroglycan, a key component linking the sarcolemma and the extracellular matrix (Chambers et al., 2003), was strongly expressed at the myosepta in homozygous mutants (data not shown) suggesting the muscle attachments are intact. The myosin and actin bands were visible in the myofibrils of wild type and homozygotes (Fig. 3H,I). Furthermore, using electron microscopy, we have confirmed that sarcomere formation was not disrupted in the mutant (Fig. 3J,K); however, the sarcomere length (distance between Z-bands) was significantly smaller in mutants (1.65±0.02 μm, *n*=2 embryos) compared with wild types (1.83±0.06 μm, *n*=3 embryos, $P<0.0001$ unpaired *t*-test). In summary, these results reveal that fibre length, lateral alignment of the myofibrils and sarcomere length are disrupted in the *nic1* mutant embryos. This phenotype shows similarity to that observed on inhibition of ryanodine receptors suggesting that during development AChR induced calcium signaling is a key regulator of slow muscle myofibril organisation.

Discussion

Neural input and activity-generated calcium signals are known to be important regulators of skeletal muscle function in the adult; however, the stages of late muscle development regulated by motor neurons in the embryo remain undefined. Our results revealed that fibre number, cell movement and sarcomere formation do not appear to be regulated via the acetylcholine receptor or calcium signalling via ryanodine receptors in the slow muscle of embryonic zebrafish. Rather our study shows that signalling via acetylcholine receptors is required for controlling myofibril and sarcomere length in the embryo. We revealed developmentally regulated calcium signalling during the initiation of neurotransmission in embryonic muscle. We propose that the characteristic patterning of intracellular calcium signals is important in the activation of specific signalling pathways in developing muscle. This possibility is currently under investigation. We conclude that motor neuron activity has a key role in defining the optimal contractile unit (sarcomere length and number) in the developing muscle. Our findings have important implications for force generation and behaviour in the embryo.

Muscle fibres are composed of bundles of myofibrils that run from one end of the cell to the other. In longitudinal section myofibrils align laterally resulting in longitudinal arrangement of sarcomeres. Our observations from the *nic1* mutant reveals that the early steps of myofibrillogenesis (Sanger et al., 2002), resulting in sarcomere formation do not appear to be controlled via nerve input. Our findings reveal that it is the organisation, most notably the shortening of the sarcomeres and the lengthening of the myofibrils, which is disrupted in the absence of nerve generated activity. Sarcomere length is one of the main factors contributing to force generation in skeletal muscle (Burkholder and Lieber, 2001). In myofibrils serial sarcomere number is adjusted to achieve the optimum sarcomere length and hence force generation (Burkholder and Lieber, 2001). Our observations that blockade of the acetylcholine receptor generated shorter sarcomeres and increased myofibril length suggest an increase in sarcomere number. Our hypothesis is that nerve activity and calcium signalling act to limit sarcomere number and therefore produce the optimal sarcomere length required for force generation during embryogenesis.

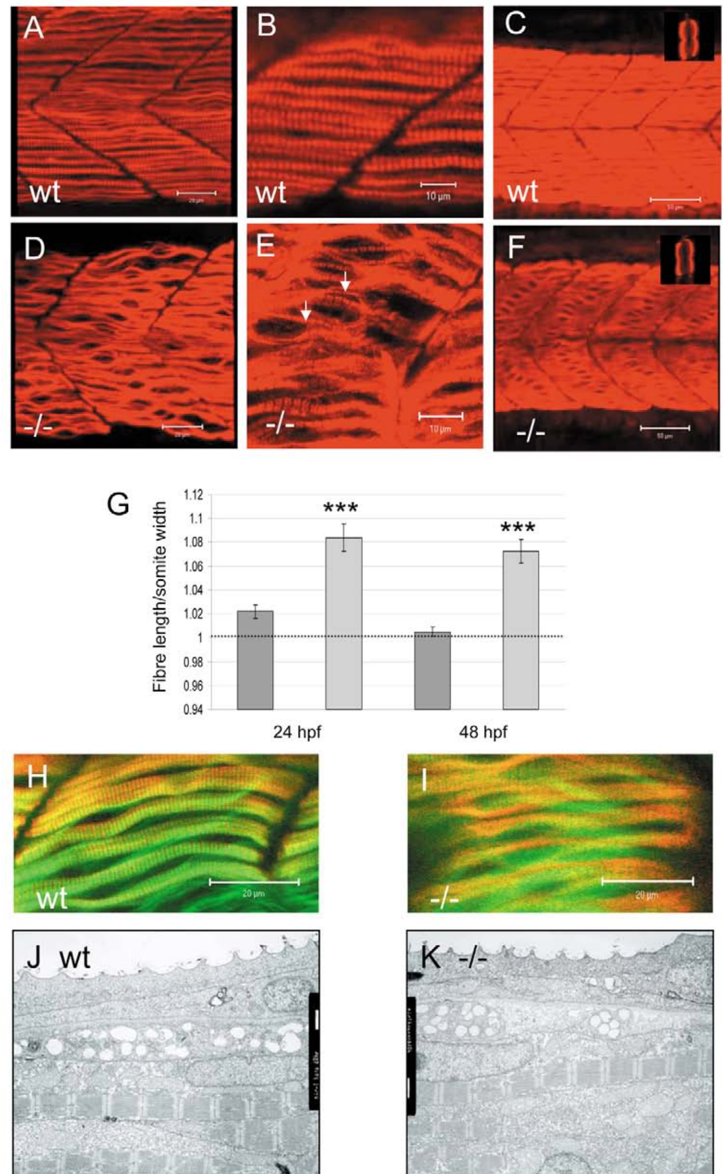

Fig. 6. Absence of acetylcholine receptors in *nic1* mutants disrupts myofibril organisation. Immunostaining with antibody F59 was performed to reveal slow muscle fibres in (A,B) 24 hpf and (C) 48 hpf wild-type embryos and (D,E) 24 hpf and (F) 48 hpf *nic1* embryos. (C,F) Insets show cross section of slow muscle myosin in anterior trunk of embryo at 48 hpf. Myofibrils organisation was disrupted in the mutants (arrows). Bars (A,D) 20 μm, (B,E) 10 μm and (C,F) 50 μm. (G) Fibre length/somite width was significantly longer in mutant embryos (light-grey bars, n=22 at 24 hpf and n=13 at 48 hpf) compared with wild-type embryos (dark-grey bars, n=24 at 24 hpf and n=26 at 48 hpf; ±s.e.m., ***P<0.0001, unpaired t-test). Dual immunostaining with phalloidin and antibody F59 revealed striations in (H) wild type and (I) homozygote *nic1* embryos at 48 hpf; bar 20 μm. Electron micrographs of longitudinal sections through axial muscles of the trunk of (J) wild type and (K) *nic1* embryos at 48 hpf. Bars, 1 μm.

Serial sarcomere number and consequently sarcomere length are important physiological parameters incorporated into the design of different muscle types. For example, in fish there is a functional specialisation of the muscle: red fibres that are slow contracting for steady swimming and white fibres for bursts of activity (Raamsdonk et al., 1982). Sarcomere length and contraction velocities in various muscle types operate at different optimal ranges in order to perform specific functions (steady swimming versus startle response) (Rome, 2002). The results presented here suggest that in the later stages of development nerve input works to refine the parameters that determine functional specialisation of the muscle. Indeed in other systems electrical activity has been implicated as the main contributing factor determining sarcomere number and optimal sarcomere lengthening (Herring et al., 1984). Our zebrafish study suggests that electrical activity regulates the sarcomere arrangement in myofibrils within embryos. The purpose of early embryonic movements in

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zebrafish, although proposed to be involved in hatching (Kimmel et al., 1974; Saint-Amant and Drapeau, 1998), has never been established. We propose that coordinated movements could be important for refining the properties (myofibril organisation) of the different embryonic muscle types. The zebrafish *nic1* mutant represents a unique vertebrate model in which to study the later steps of activity-dependent myofibrillogenesis during development in vivo.

Our study has revealed that acetylcholine and calcium signalling can control myofibril organisation; however, downstream steps of the pathway remain to be determined. Intermediate filaments, such as desmin (Li et al., 1997), making up the exosarcomeric cytoskeleton are responsible for the alignment of myofibrils. Evidence, from studies in desmin knockout mice after hindlimb immobilisation, has revealed a role for desmin in the regulation of sarcomere number in different skeletal muscle types (Shah et al., 2001). Interestingly binding of the calcium-modulated proteins, S100A1 and S100B, results in the inhibition of desmin assemblies and disassembly of preformed desmin (Garbuglia et al., 1999). Garbuglia and colleagues (Garbuglia et al., 1999) suggest that an elevated intracellular calcium concentration promotes interaction of S100B with desmin causing intermediate filament disassembly in vivo. Our observations using the *nic1* mutant reveal that the lack of activity dependent calcium signalling resulted in increased myofibril and decreased sarcomere length. This is consistent with a model whereby acetylcholine generated calcium signals promote S100A1 and S100B and desmin interaction thereby causing intermediate filament disassembly and regulation of sarcomere number and myofibril organisation.

In summary, our study demonstrates a role for nerve activity and downstream intracellular calcium signals in the regulation of myofibril organisation during embryogenesis. Myofibrillogenesis and the organisation of the contractile units (sarcomeres) are critical for proper muscle function in both the embryo and adult. An important future step will be the identification of other signalling proteins involved in activity regulated fibre formation in the embryo.

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